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THE PROBLEM OF THE DETERMINATION OF SECTION THICKNESS

[Following is the translation of a German-language article by S. Weissbach, Pathological Institute of Frankfurt/Main University, published in Acta Histochemica, No. 9, 1960, pages 183-187.]

(Note: Investigation carried out with the support of the German Research Association.)

For the exact determination of substance concentration and volume as carried out with microspectrographic and microradiographic quantitative methods, knowledge of the absolute thickness of biological sections is necessary. Attempts have been made to solve this problem in various ways by utilizing the following methods:

1. Focusing on upper and lower face of object;
2. Weighing of section;
3. Direct measurement by means of the ocular micrometer;
4. Interference methods;
5. Reflection of beta particles;
6. Diamond method;
7. Stereoscopic method;
8. Direct measurement with precision measuring instrument.

The focusing method was based on the Abbe and on the Berek theory of depth of focus and used objectives with high capability of vertical resolution. The vertical movement of the tube was read from the micrometer screw of the microscope. However, it was found that the raising and lowering of the tube (in earlier microscopes, the precision adjustment acted on the tube) was not a linear function and made very exact work impossible. In 1954, Lance and Engstroem carried out measurement of thickness of biological objects by the focusing method with a very highly developed microscope and found errors of 10-13 per cent.

When weighing the section, it is possible at most to determine an average section which is not representative of the location investigated. Moreover, by mathematical division of a paraffin cube in sections of 5/u, no particular consideration is given to the density of the tissue in individual parts of the sections.

We carried out some weighing of sections on paraffin cubes without tissue. The cubes had been cooled for a certain length of time and were cut with a microtome adjusted to 5/u under uniform external conditions (refrigerated chamber). The weights show a spread of 5 %. This figure indicates the reproducibility of the microtome under ideal conditions but indicates unfortunately nothing for absolute thickness.

In 1957, Merriam bent a part of the section on an aluminum foil upward at right angle, by adroit manipulation, and so made the free edge accessible for direct measurement.

Various investigators (Davies, Wilkins, Mellors, Menzel and Schmidt, cf. Hallen) utilized the interference microscope for determination of thickness. However, the refraction index of the tissue must be known for this type of determination. In some cases, the method is difficult to apply because different object structures frequently have a different refraction index.

In electron-microscopics, Gotthardt developed the stereoscopic method. This enabled him to determine thicknesses between 0.1-1.0 /u. The method is unfortunately not simple for sections of 5/u and therefore is not applicable to our cell-analytic investigations. Baehr utilized, for determination of thickness, the impression of a Vickers or Knoop diamond which makes possible mathematical determination of thickness from its known angles and measured distances. Glimstedt and Hakansson carried out direct measurement by reducing the operating pressure of a "microkator" (a precision measurement instrument of the type of the German Millimess, making possible measurements of object with a thickness of 0.1/u) to 200 mg and so determined thickness directly.

Odeblad (1957) attempted to solve this problem in a simple manner by utilizing the reflection of energy-poor beta particles. As emitting substance, he used labeled C^{14} and S^{35}.

Hallen finally returned to the focusing method. Instead of the standard microscope, he utilized a top-illumination microscope and measured the motion of the fine adjustment with a mikrokator and not with a micrometer screw. In order to circumvent the difficulties in the determination of the upper and lower face of the section,

he installed a wire of 30 or 100/u in place of the light-field diaphrame in the Koehler lighting system which is then projected on the upper and lower face of the section and so appreciably facilitates their recognition. We here in the Institute also turned to the focusing method and utilized a phase-contrast microscope instead of the top-illumination microscope. It is highly suitable for recognition of the boundary surfaces of the section and, with some practice, 50 measurements in the same location produce an error of 2.28 %. The vertical movement of the object table with the micrometer screw is transmitted to a Mikrokator which is rigidly attached to the microscope by a strong metal clamp. The entire aggregate of microscope, Mikrokator and metal clamp are mounted on a steel base embedded in a concrete table and so protected from mechanical influences. For the measurements, a binocular drawtube with an ocular magnification of (?) 12.5, a phase-contrast objective with a magnification of (?) 40 (N.A. 0.65) and (?) 100 (N.A. 1.25) was utilized.

Thyroid colloid, hyaline cartilage and liver tissue was utilized for the measurements. In order to control the determination of section thickness by the focusing method, we simultaneously carried out comparative determination of section with the interference microscope. If a section prepared with a microtome adjusted to 5/u was measured by the focusing method with an objective of N.A. 0.65 and with an objective of N.A. 1.25 at the same location, this produced a difference of about 2.5-3.0/u. For example, a thickness of 4.96/u is measured with N.A. 0.65 and a thickness of 7.39/u with N.A. 1.25. This difference was repeated through the entire series of sections, was correspondingly smaller for sections of 2 and 3/u but always recurred with minor deviations. This discrepancy must therefore find its origin in the optical system and not in the observer or the measuring equipment.

A simple trigonometric consideration will produce the conclusion that the absolute thickness of the measured image projection, multiplied by the quotient of the tangent of the angle of the incident ray to the perpendicular and the tangent of the angle of the refracted ray to the perpendicular, is expressed by the following formula

$$d = h \cdot \frac{tga}{tgB} = \cdot (tga \cdot ctgB)$$

Because the angles are relatively large, tan cannot be equated here to sin. If we enter the corresponding values in the aperture formula $A = n \cdot \sin \alpha$, we then obtain an angle of 40.5° for an objective of N.A. 0.65 and an angle of 55.5° for an objective of N.A. 1.25. If we therefore want to determine tan α and cotan β, we must go back to the aperture formula and enter the value of the embedding medium or the object for the respective n.

This results in the necessity of determining the refraction index of the object. For this we utilize liquids with a defined refractice index of 1.46-1.64 with a graduation of 0.01. This puts us in position to accurately determine the refractive index to the second decimal figure by approximation; the third decimal figure can then be found with relative accuracy by interpolation. A more exact determination is not easily possible and not necessary because greater accuracy exceeds the possibility of the focusing method. In measurements of refractive indices, we proceeded by utilising some section ahead of and some beyond the sections to be measured. In doing so, no difference was shown even in the third decimal place. We then used this refractive index for our formula and also for the determination of thickness with the Baker interference microscope.

In accordance with the explanations above, we considered the N.A. of the objectives and so found satisfactory concordance of the values of both objectives. The measurement of the thickness of the section at the same location with the interference microscope also showed satisfactory concording values within 2 %. For the (?) 100 objective with greater depth of focus, the values are even better.

It results from these measurements that, in the determination of thickness, we absolutely must also consider the N.A. of the objective and the refractive index of the tissue. Without these values, the findings obtained will always be inexact. The refractive index may occasionally be rather difficult to determine, e.g. in the liver, in which structures with another refractive index occur.

Discussion:

Piller: The accuracy of measurement of thickness of focusing on upper and lower face depends, among other factors, on the range of depth of focus which is given in turn by the image scale and the effective numerical aperture. Moreover, the quality of the image-forming optics plays a role. It is therefore preferable to carry out tests and/or calibration measurements in which the microscope is accurately adjusted as later in practical work in order to control the difference of height between the adjustment on the upper face and that of the lower face by a Mikrokator.

Schiener: The determination of section thickness with the Baker Interference microscope (Shearing system, fringe-field ocular), was carried out by us with the following equation:

$$d = \frac{\lambda}{(n_0 - n_m)} \cdot \frac{1}{n} \sum_{1}^{n} \frac{(d_a \cdot n_a / D)}{d_a} \quad 1 \; [cm].$$

In this formula, (d) = average thickness of section, λ = wave length of monochromatic light, (n_o (?) n_p) = difference of refractive indices of object and surrounding medium, (n) = number of measuring points over the length of the band deflection investigated. The interval of the bands from each other is (d_a) and (d_a x n_a) is the magnitude of deflection in whole band-interval units. (D) determines the interval of the deviation from the first reference band and (i) defines that part of the equation which is to be summated. This method of determination can be carried out with the Baker interference microscope in the range free of double image. With a 100-x objective, this range is limited on one side to 27/u and with the 40-x objective to 160/u.

Casdersson: I believe that the problem of the determination of thickness represents a great difficulty. The Baeur method was developed in order to circumvent the unreliability of the determination of refractive indices. Biological problems should be formulated as far as possible so that whole cells can be utilized (smears) which avoid all these difficulties.

Sandritter: For purely comparative measurement, the problem can be generally bypassed by bringing various objects in one paraffin cube.

Trapp: I regard as a disadvantage of these very excellent mutually confirmatory findings that they are all dependent on the determination of refractive index which brings on the other hand a lack of reliability in the final results which it is difficult to control. I should like to suggest some attempts at eliminating the determination of refractive index, e.g. by transforming measurement of depths into measurement of laberal extent.

Weissbach: I should like to stress that the test objects utilized in my investigations have a very homogenous refractive index (thyroid colloid). Further investigation will be needed to check for the amount of error which differences of refractice index within different structures occurring in the same tissue, introduce into the measurement of thickness.

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